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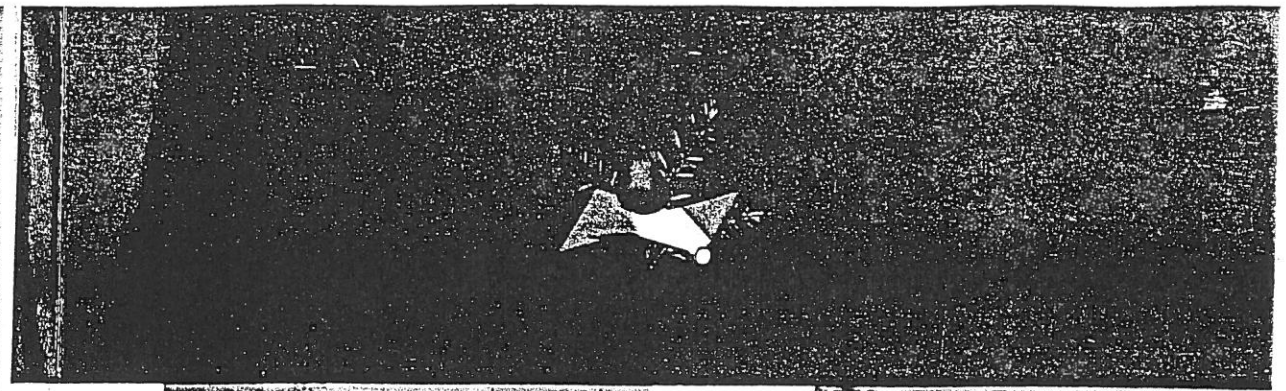
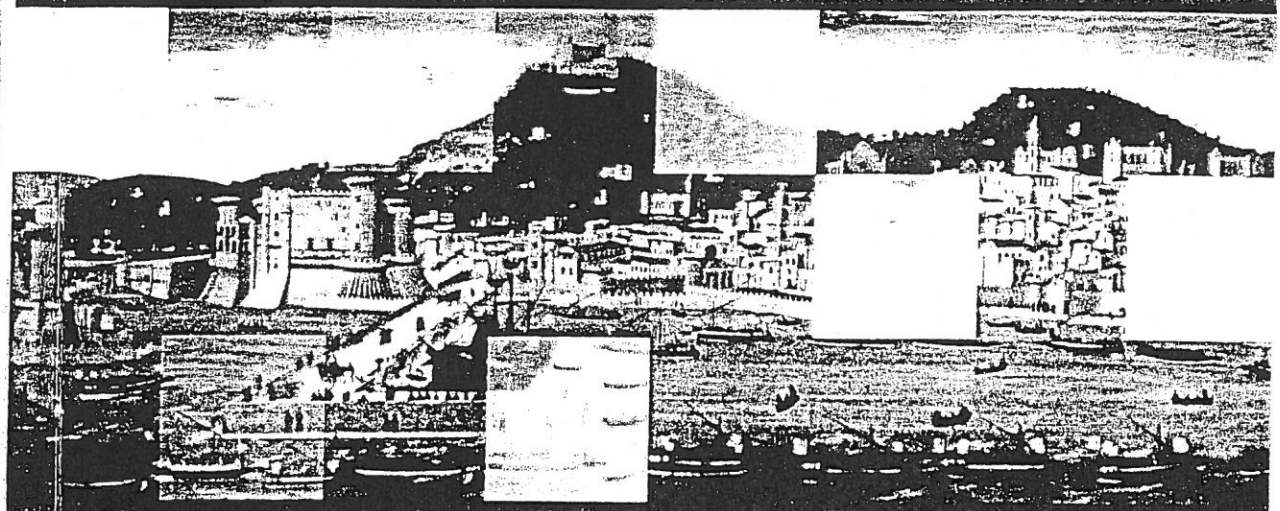
Publication date:
2009

Document version
Publisher's PDF, also known as Version of record





8th Carbohydrate Bioengineering Meeting

PROGRAM AND ABSTRACTS

May 10-13, 2009 Ischia, Naples, Italy

Expression and binding properties of CBM45 from *Solanum tuberosum*

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Starch binding domains (SBDs) have been found in CBM families 20, 21, 25, 26, 34, 41, 45, 48 and 53 [1]. Recently a new family of SBDs has been discovered. [2] Family CBM45 contains only sequences of intra-cellular domains from the plant kingdom. The currently known 25 sequences contain tandem domains which reveal a surprisingly low sequence similarity of 25% between the domains. CBM45 is found as an N-terminal tandem domain in plastidial amylases (EC 3.2.1.1) and glucan water dikinases (GWDs, EC 2.7.9.4). GWD1 is expressed in the photosynthetic active parts of the plant and phosphorylates transient starch in the plastids.
In order to probe the domain borders several single CBM45 constructs of GWD1 from *Solanum tuberosum* (potato) were made and expressed as cleavable His-fusion protein in *E. coli*. Protein integrity was tested after removal of the His-tag by differential scanning calorimety (DSC), revealing high thermo-stability of CBM45-2 (T_m of 84°C) and reversibility of protein folding. Binding properties of the CBM45 were tested by ITC and Biacore with soluble low molecular weight starch mimic motifs.

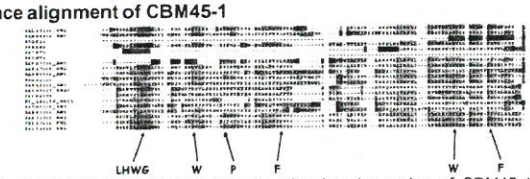
CBM45 are found in Glucan water dikinases and plastidial amylases

The CBM45 family is one of the smallest CBM families. CBM45 are exclusively found in plants and only in two classes of proteins, in glucan water-dikinases (GWD) and in plastidial α-amylases. Plants have three GWDs, two of them have CBM45s. Most CBM45-protiens are localized in the plastide as they have a putative transit peptide.
GWD1 from *solanum tuberosum* is redox-regulated, the reduced form is inactive, the oxidized form is active during the night, phosphorolating transient starch and triggering rapid degradation.

Domain organization

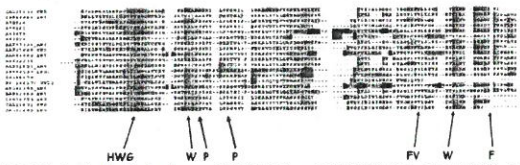
CBM45 are found exclusively in the N-terminus of plant proteins, and always as tandem domain. The CBMs are separated by approximately 300 amino acids of unknown function. Identity of CBM45-1 and CBM45-2 of the GWD3 from *Solanum tuberosum* is 20% low. A characteristic pattern of three tryptophan amino acids can be found in all CBM45.

Sequence alignment of CBM45-1



Shortened alignment of selected sequences, showing the region of CBM45-1. In the N-termini putative transit peptides for plastid localization can be found. Yellow to red color indicates regions moderate to high similarity, green to blue indicates regions low to lack of similarity.

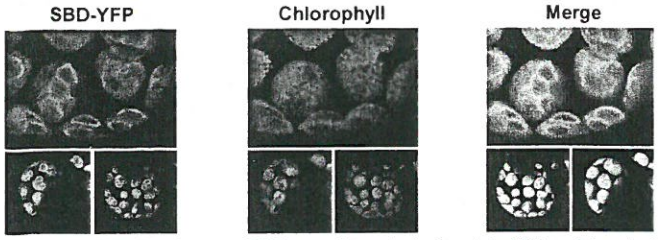
Sequence alignment of CBM45-2



Region of CBM45-2, similarity between CBM45-1 and CBM45-2 is about 45%, identity at 20 %. The catalytic domain continues after CBM45-2 without a linker.

In vivo cellular localization of CBM45 by CLSM

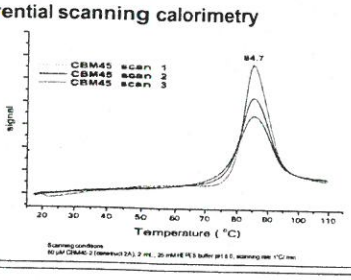
Transient expression of a CBM45-YFP fusion in tobacco leaf mesophyll cells was done to confirm translocation of CBM45 into the plastid. YFP fluorescence (green), chlorophyll autofluorescence (red) and a merged image of the two channels are shown.



The complete CBM45 of potato GWD was C-terminally fused to YFP and transiently expressed in *Nicotiana benthamiana* leaves by infiltration with *Agrobacterium tumefaciens*. Expression and localization was investigated by confocal laser scanning microscopy (CLSM). Binding was observed to granular structures in the chloroplasts. Some fluorescence was also observed in the chloroplast stroma, possibly as a consequence of the suggested weak binding affinity of the SBD.

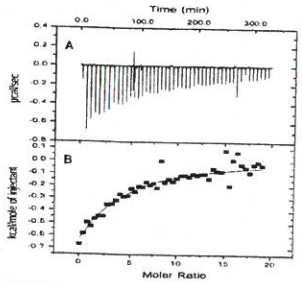
Protein stability evaluation by differential scanning calorimetry

The potato CBM45-2 was tested after proteolytic removal of the N-terminal hexahistidine-tag. Protein unfolding was tested by differential scanning calorimetry (DSC) in the temperature range form 15°C to 115 °C. As shown by three consecutive DSC runs, CBM45-2A refolds upon cooling.
CBM45-2A shows a surprisingly high unfolding temperature of 84.7°C.



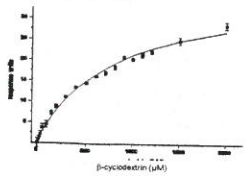
Preliminary binding studies by isothermal calorimetric titration

Binding properties of CBM45-2 were tested by isothermal calorimetric titration (ITC) with β-cyclodextrin as a soluble starch mimic motif. Panel A shows the ITC raw data, detecting the heat generation per injection. Panel B shows the peak areas, fitted to a one binding site model, red points were not considered in the curve fitting.
Due to the limited solubility both of the CBM45-2 and the ligand only a part of the expected hyperbolic binding curve can be obtained. The data were fitted with a single site binding model and the K_d was ~400 µM. The weak binding suggests a reversible binding under native conditions.



Preliminary binding studies by surface plasmon resonance

CBM45	pH	ligand	K_d mM	R_{max}	χ^2	Data points
CBM45-1	7	β-cd	0.35	70.7	3.5	8
	8	β-cd	0.19	45.3	2.9	8
CBM45-2	7	β-cd	0.38	59	0.7	24
		α-cd	0.39	53	1.8	24
	8	β-cd	0.42	67	2.7	24
		α-cd	0.29	48	1.5	24



Surface plasmon resonance (SPR) has been employed to test a broader spectrum of starch mimic motif. CBM45 proteins were biotinylated and immobilized on a streptavidin chip surface. α, β, γ–cyclodextrin, 6-O-α-d-glucosyl– and 6-O-α-d-maltosyl-cyclodextrin were tested as ligands. None of the tested cyclodextrins showed tighter binding to either CBM45-1, CBM45–2 or to the full N-terminal region. Cyclodextrins show in SPR experiments binding with comparable affinity constants as in the ITC experiments. Increasing concentrations of sodium chloride have very little influence on binding, suggesting a small contribution of ionic interactions.

Conclusions

CBM45-2 is an exceptionally thermostable protein for a mesophile. This indicates that the isolated CBM45 is structurally intact. Binding to starch mimic motifs reveals a rather mediocre binding affinity (K_d ~400µM). Considering the biological function of glucan water dikinases in phophorylation of transient starch during the night and thereby inducing the depolymerisation, a strong binding to starch would be a hindrance for enzyme regulation during the diurnal cycle. The exact influence of CBM45 on catalytic activity remains to be studied. Plastidial α-amylases containing a CBM45 tandem domain are with simple activity measurements an ideal target for further research.

Acknowledgements

A Hans Christian Ørsted postdoctoral fellowship from DTU for MJB is gratefully acknowledged. The financial support from the Carlsberg foundation is also gratefully acknowledged. A Novo scholarship to JMA is thankfully acknowledged.

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